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Relationship of TT Virus Infection With Prevalence of Hepatitis C Virus Infection and Elevated Alanine Aminotransferase Levels

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A novel DNA virus, TT virus (TTV), was identified in a Japanese patient with posttransfusion hepatitis. The epidemiology and etiological role of this virus have not been elucidated. We investigated the epidemiology of TTV infection in hepatitis C virus (HCV) high endemic and low endemic areas, R town and M town, respectively. The seroprevalence, potential risk factors, and laboratory features of TTV in relation to those of HCV were analyzed. TTV DNA was detected using a seminested polymerase chain reaction and the TTV genotypes were determined by a direct sequencing method. TTV DNA was detected in 16.1% of the subjects in R town and 17.5% of those in M town. The TTV DNA positivity rates of the 2 areas did not differ significantly. A history of blood transfusion was not a specific risk factor for TTV infection. The mean serum alanine aminotransferase (ALT) level of the anti-HCV-positive subjects was significantly higher than that of the TTV DNA-positive subjects, most of whom had normal ALT levels. The TTV genotype distributions of these 2 distinct areas differed. These results suggest that TTV infection is widespread with a geographical genotypic distribution independent of HCV infection and that the ALT abnormalities are not attributable to TTV but to HCV infection in the general population. ***J. Med. Virol. 58:235–238, 1999.***

KEY WORDS: TTV; HCV; hepatitis; epidemiology; HGV

INTRODUCTION

Recently, a novel DNA virus, TT virus (TTV), was identified in the serum of a patient who had posttransfusion hepatitis but was negative for all the conventional hepatitis markers [Nishizawa et al., 1997]. TTV is a single-stranded linear DNA virus consisting of longer than 3,739 nucleotides, and its virion is believed to be unenveloped [Okamoto et al., 1998a]. The TTV genome varies widely depending on the strain, and at least 4 genotypes have been classified [Höhne et al., 1998; Okamoto et al., 1998a]. The distribution of TTV is worldwide, and blood donors or patients with liver diseases show a variety of TTV positivity rates among nations [Biagini et al., 1998; Charlton et al., 1998; Höhne et al., 1998; Naoumov et al., 1998; Okamoto et al., 1998a; Simmonds et al., 1998]. This virus may provide important clues to the etiology of cryptogenic liver diseases. However, TTV DNA was detected with a high frequency not only in hepatitis patients lacking serologic markers, but in the general population of blood donors [Okamoto et al., 1998a]. Therefore, it is important to carry out further investigations on the pathogenesis of TTV. Furthermore, the epidemiological significance of this virus with respect to public health has not been well understood.

In this study, we investigated the epidemiology of TTV infection, including its prevalence, the risk factors for infection, and the viral genotypic distribution in the general populations of 2 distinct areas. In order to analyze the liver enzyme abnormalities due to TTV infection and their relationships to hepatitis C virus (HCV) infection, the serum alanine aminotransferase (ALT) levels of randomly selected subjects living in these two HCV high endemic and low endemic areas, which have been reported previously [Ishibashi et al., 1996], were compared. Such an approach would further the understanding of the epidemiology of TTV infection and clarify the etiological role of TTV in liver dysfunction in the general population and its relationship to HCV infection.

MATERIALS AND METHODS

Subjects

We conducted a mass survey of the liver function tests by analyzing sera of the residents of 6 towns in Yamagata Prefecture in Japan from 1991 to 1993 [Ishibashi et al., 1996]. This survey revealed a marked re-

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TABLE I. Subject Characteristics

	R town	M town	*P*
Number (male)	304 (159)	114 (36)	
Age (years)[a]	60.2 ± 12.8	62.7 ± 11.0	NS[b,d]
ALT (IU/l)[a]	32.2 ± 37.3	21.9 ± 13.4	<0.001[b,e]
Blood transfusion (%)	26 (8.6)	5 (4.2)	NS[c,d]

[a]Mean ± SD.
[b]Student's *t*-test.
[c]Chi-squared test.
[d]NS, not significant.
[e]Significant difference in ALT level between 2 towns.

gional accumulation of HCV infection. In this study, 2 distinct areas, R town and M town, were selected according to the frequencies of anti-HCV antibody (anti-HCV) positivity. The residents of R town had a high frequency for positive anti-HCV (810/2,915; 27.8%) and those of M town had an average frequency (99/3, 194; 3.1%). The serum samples negative for hepatitis B surface antigen were collected randomly, and 304 and 114 samples of towns R and M, respectively, were tested for both anti-HCV and TTV DNA. The characteristics of the subjects from both towns are shown in Table I.

Assay for HCV Marker

The second-generation enzyme-linked immunosorbent kit (Abbott HCV EIA 2.0, Dainabot, Tokyo, Japan), was used to test for anti-HCV.

Detection of TTV DNA

Serum samples were stored at −80°C before testing. DNA was extracted directly from 200 μl serum using the QIAamp Blood Kit (Qiagen Ltd., Hilden, Germany), redissolved in 30 μl water, and a 5 μl aliquot of this solution was used for the polymerase chain reaction (PCR). TTV DNA was amplified by a seminested PCR using primers derived from the conserved regions, as reported previously [Okamoto et al., 1998a]. For the first round of PCR, 25 μM sense primer (5′ACAGACAGAGGAGAAGGCAACATG3′) and 25 μM antisense primer (5′CTGGCATTTTACCATTTCCAAAGTT3′) were mixed with 50 μl PCR solution comprising 2.5 mM deoxynucleotide triphosphate mixture, 5 U/l AmpliTaq DNA Polymerase (Roche Molecular Systems, Branchburg, NJ), and PCR buffer. The PCR was carried out for 35 cycles at 94°C for 20 sec, 60°C for 20 sec, and 72°C for 60 sec, with an additional extension step at 72°C for 7 min using the Perkin-Elmer (Oak Brook, Norwalk, CT) 9600 thermal cycler. For the second round of PCR, 2.5 μl first PCR product was subjected to 25 amplification cycles with the sense primer (5′GGCAACATGTTATGGATAGACTGG3′) and the above antisense primer. A 271 bp PCR product was detected by electrophoresis on a 2% agarose gel followed by staining with ethidium bromide. In order to confirm the specificity of the PCR results, all of the samples were subjected to repeat analysis.

TABLE II. Prevalence of Anti-HCV and TTV DNA in Towns High Endemic (R Town) and Low Endemic (M Town) for HCV

	R town	M town	*P*[a]
Anti-HCV (%)	91/304 (29.9)	2/114 (1.8)	<0.001[b]
TTV DNA (%)	49/304 (16.1)	20/114 (17.5)	NS[c]

[a]Chi-squared test.
[b]Significant difference in anti-HCV positivity between 2 towns.
[c]NS, not significant.

Direct Sequencing of the TTV

The amplified TTV DNA was purified using a QIAquick PCR purification kit (Qiagen Ltd.). Direct sequencing of the partial TTV DNA genome was performed by the fluorescent dye terminater cycle method using the ABI 310 automated sequencer (Applied Biosystems, Forster City, CA). Genotyping was performed according to the classification system proposed by Okamoto et al. [1998a].

Statistical Analysis

The categorical and continuous variables were compared using the chi-squared test and Student's *t*-test, respectively. Both tests were 2-sided. The results were expressed as means ± SD. Differences at values of $P < 0.05$ were considered to be significant.

RESULTS

Prevalence of Anti-HCV and TTV DNA in the HCV High Endemic and Low Endemic Areas

Rates of positivity for anti-HCV and TTV DNA in 2 areas are shown in Table II. In this study, the prevalence of anti-HCV in the high HCV endemic "R town" was 91/304 (29.9%) and that in the low endemic "M town" was 2/114 (1.8%, $P < 0.001$). The positivity rates of TTV DNA were 49/304 (16.1%) and 20/114 (17.5%) in towns R and M, respectively. The prevalences of TTV DNA in the 2 towns were almost the same. In order to clarify whether TTV and HCV infection occurred together in the same individuals, the TTV DNA positivity rates of the subjects in R town who were anti-HCV positive and those who were anti-HCV negative were compared. There was no significant difference in the TTV DNA positivity rates between the subjects positive for anti-HCV (17/91; 18.7%) and those negative for anti-HCV (32/213; 15.0%) (Table III). These results suggest that TTV infection is widespread in these 2 distinct areas independently of HCV infection.

Prevalence of Anti-HCV and TTV DNA in Relation to a History of Blood Transfusion

Generally, exposure to blood products is considered to be one of the major risk factors for viral transmission [Nishioka et al., 1991]. Relationship between TTV infection and a history of blood transfusion was analyzed. The results are shown in Table IV. As we reported previously [Ishibashi et al., 1996], the positivity rate of anti-HCV was closely related to a history of blood transfusion (15.1% with vs. 5.2% without; $P < 0.01$).

TABLE III. Prevalence of TTV DNA With or Without Anti-HCV in R Town

	Anti-HCV		
	Positive	Negative	P^a
TTV DNA (%)	17/91 (18.7)	32/213 (15.0)	NS[b]

[a]chi-squared test.
[b]NS, not significant.

However, the positivity rate of TTV DNA was not associated with a history of blood transfusion (10.1% with vs. 6.9% without; not significantly different).

Relationships Between Abnormal Liver Function Tests and Hepatitis Virus Markers

The rates for ALT abnormality (ALT value > 35 IU/l) according to TTV DNA and anti-HCV status in R town are shown in Table V. The subjects positive for anti-HCV only had a higher rate for ALT abnormality than those positive for TTV DNA only (44.6% vs. 15.6%; $P < 0.01$). There was no significant difference in the rates for ALT abnormality between those positive for TTV DNA only and those negative for both TTV DNA and anti-HCV. The mean serum ALT levels of the 74 subjects positive for anti-HCV only and the 17 positive for both TTV DNA and anti-HCV were 44.1 ± 33.5 and 42.5 ± 25.0 IU/l, respectively. Both values were significantly higher than those of the 32 subjects positive for TTV DNA only (25.6 ± 13.6 IU/l; $P < 0.01$) and the 181 negative for both TTV DNA and anti-HCV (29.7 ± 14.2 IU/l; $P < 0.01$). Most of the 32 subjects positive for TTV DNA only had normal ALT levels. These data suggest that the major causative agent associated with the abnormal liver function tests was not the TTV, but the HCV endemic in the general population.

Genotype Variations in 2 Distinct Areas

The partial sequence of the TTV DNA amplified by the PCR was determined and compared with the reported sequence. According to the genotype classification proposed by Okamoto et al. [1998a], the TTV DNA was classified into 2 groups, 1 (G1) and 2 (G2). G1 and G2 were divided into 2 subgroups, G1a and G1b, and G2a and G2b, respectively. Seven of the 14 samples from R town were classified as G1 (2 G1a and 5 G1b) which showed 1–5% nucleotide divergence, and the other 7 were G2 (5 G2a and 2 G2b) with 1–31% divergence. Sixteen samples from M town were analyzed: 14 were classified as G1 (6 G1a and 8 G1b) with 2–7% divergence, and the other 2 as G2 (1 G2a and 1 G2b) with 3–14% divergence. G1 isolates of TTV occurred significantly more frequently in subjects from M town than R town (14/16, 87.5% vs. 7/14, 50%; $P < 0.05$).

DISCUSSION

In this study, we demonstrated, for the first time, the prevalence of TTV in the hepatitis C high endemic area (R town) in comparison with that of the hepatitis C low endemic area (M town), and analyzed the cause of abnormal liver function tests in the general population. TTV DNA was detected in subjects of both towns irrespective of the positivity for anti-HCV, and no regional accumulation of TTV infection was found. This is interesting because the infection of the 2 viruses may have occurred in the same individual through different routes. Our previous report on the epidemiology of hepatitis G virus (HGV) in the same towns revealed that HGV infection was detected frequently in subjects with HCV infection [Zhang et al., 1998]. We found in this study that, unlike HGV, the transmission of TTV was not related to HCV infection. The transmission route of TTV has not been clarified. As TTV was first isolated from the serum of a patient with posttransfusion hepatitis, parenteral transmission was considered to be most likely [Nishizawa et al., 1997; Okamoto et al., 1998a]. A history of prior exposure to blood products appears to increase the risk of TTV infection. The individuals positive for anti-HCV had a higher frequency of a history of blood transfusion than those negative for anti-HCV, but we found no strong evidence that a history of blood transfusion was closely associated with the transmission of TTV. A previous study showed that TTV DNA was detected in more than 10% of Japanese blood donors [Okamoto et al., 1998a]. The positivity rates of TTV DNA in the general population of 2 distinct areas in this study are consistent with those of the Japanese blood donors. These findings led us to hypothesize that TTV is transmitted not only by a parenteral route but by a non-parenteral route [Okamoto et al., 1998b]. Further epidemiological studies are in progress to show how TTV is transmitted and spreads among humans.

The etiological importance of TTV in association with liver diseases has not been characterized [Cossart, 1998]. Previous studies suggested that TTV infection could cause a hepatitis with elevated ALT levels [Nishizawa et al., 1997; Okamoto et al., 1998a]. However, the reason why there are so many healthy carriers in such a general population as blood donors remains unknown. A recent study revealed that most TTV DNA-positive subjects had normal ALT levels with minor histological changes [Naoumov et al., 1998]. Our study is the first to demonstrate the prevalence of TTV infection in the general population in relation to HCV infection, which is the most important cause of chronic liver disease in Japan. We found that TTV infection is widespread with a high prevalence of over 15% in the general population, but the ALT abnormality is due to HCV infection. This suggests that HCV infection plays an important etiological role in the abnormal liver function tests occurring in the general population. A minority of TTV DNA-positive individuals had abnormal ALT levels, although the frequency was lower than that of those negative for both TTV DNA and anti-HCV. Alcohol intake, nutritional factors, or unknown agents might be involved in such cases.

TTV has been reported to show genotype variations [Höhne et al., 1998; Okamoto et al., 1998a; Tanaka et al., 1998]. The different genotypic variation profiles of the 2 towns may explain the regional distributions of

TABLE IV. Prevalence of Anti-HCV and TTV DNA in Relation to History of Blood Transfusion

Blood transfusion	Anti-HCV (%)			TTV DNA (%)		
	Positive	Negative	P^a	Positive	Negative	P^a
R town	14/91 (15.4)	12/213 (5.6)	<0.01[b]	6/49 (12.2)	20/255 (7.8)	NS[c]
M town	0/2 (0)	5/112 (4.5)	NS	1/20 (5.0)	4/94 (4.3)	NS
Total	14/93 (15.1)	17/325 (5.2)	<0.01[b]	7/69 (10.1)	24/349 (6.9)	NS

[a]Chi-squared test.
[b]Significant difference between anti-HCV positivity and history of blood transfusion.
[c]NS, not significant.

TABLE V. ALT Abnormality With or Without Anti-HCV and TTV DNA

Anti-HCV	TTV DNA	
	Positive	Negative
Positive	9/17 (52.9)	33/74 (44.6)
Negative	5/32 (15.6)	51/181 (28.2)

different viral genotypes. The variability of the TTV DNA positivity rates among nations and areas may have arisen because the primers used for the PCR were not suitable for the detection of other isolates [Takahashi et al., 1998]. Further molecular biological studies on TTV could provide important information to resolve these problems.

In conclusion, this study showed that HCV permeability in the general population is of epidemiological significance in association with liver dysfunction, and that TTV is widespread independently of HCV infection.

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